Available online at www.sciencedirect.com

ScienceDirect

Journal of Virological Methods 147 (2008) 118–126

Journal of Virological Methods

www.elsevier.com/locate/jviromet

Oligonucleotide microarray-based detection and genotyping of *Plum pox virus*

Graziella Pasquini [a], Marina Barba [a,*], Ahmed Hadidi [b,1], Francesco Faggioli [a], Rodolfo Negri [c], Iris Sobol [d], Antonio Tiberini [a], Kadriye Caglayan [e], Hamed Mazyad [f], Ghandi Anfoka [g], Murad Ghanim [h], Mohammad Zeidan [i], Henryk Czosnek [d]

[a] *CRA-Istituto Sperimentale per la Patologia Vegetale, Via C.G. Bertero 22, 00156 Rome, Italy*
[b] *Agricultural Research Service, United States Department of Agriculture, Beltsville, MD 20705, USA*
[c] *Laboratorio di Genomica Funzionale e Proteomica dei Sistemi Modello, Dipartimento di Biologia cellulare e dello Sviluppo, Università degli Studi La Sapienza, 00185 Rome, Italy*
[d] *Institute of Plant Sciences and Genetics in Agriculture, Faculty of Agriculture, The Hebrew University of Jerusalem, Rehovot 76100, Israel*
[e] *Department of Plant Protection, Faculty of Agriculture, Mustafa Kemal University, 31034 Antakya-Hatay, Turkey*
[f] *Plant Pathology Research Institute, Agricultural Research Center, Ministry of Agriculture and Land Reclamation, Giza 12619, Egypt*
[g] *Department of Technology, Faculty of Agricultural Technology, Al-Balqa' Applied University, Al-Salt 19117, Jordan*
[h] *Department of Entomology, Agricultural Research Organization, The Volcani Center, Bet Dagan 50250, Israel*
[i] *Plant Protection and Inspection Services, Ministry of Agriculture, Bet Dagan 50250, Israel*

Received 16 May 2007; received in revised form 3 August 2007; accepted 22 August 2007
Available online 24 October 2007

Abstract

Plum pox virus (PPV) is the most damaging viral pathogen of stone fruits. The detection and identification of its strains are therefore of critical importance to plant quarantine and certification programs. Existing methods to screen strains of PPV suffer from significant limitations such as the simultaneous detection and genotyping of several strains of PPV in samples infected with different isolates of the virus.

A genomic strategy for PPV screening based on the viral nucleotide sequence was developed to enable the detection and genotyping of the virus from infected plant tissue or biological samples. The basis of this approach is a long 70-mer oligonucleotide DNA microarray capable of simultaneously detecting and genotyping PPV strains. Several 70-mer oligonucleotide probes were specific for the detection and genotyping of individual PPV isolates to their strains. Other probes were specific for the detection and identification of two or three PPV strains. One probe (universal), derived from the genome highly conserved 3′ non-translated region, detected all individual strains of PPV. This universal PPV probe, combined with probes specific for each known strain, could be used for new PPV strain discovery. Finally, indirect fluorescent labeling of cDNA with cyanine after cDNA synthesis enhanced the sensitivity of the virus detection without the use of the PCR amplification step.

The PPV microarray detected and identified efficiently the PPV strains in PPV-infected peach, apricot and *Nicotiana benthamiana* leaves. This PPV detection method is versatile, and enables the simultaneous detection of plant pathogens.

Keywords: Diagnosis; Genotyping; Microarrays; 70-mer oligonucleotide probe; *Plum pox virus*; PPV strains; PPV-D; PPV-M; PPV-C; PPV-EA

1. Introduction

Plum pox virus (PPV), the causal agent of sharka disease, is a member of the genus *Potyvirus* and the family *Potyviridae*. PPV is the most damaging pathogen of stone fruit trees; it has been responsible for serious losses to apricot, plum, peach, and possibly cherry orchards. Yield losses may reach 90–100% in susceptible plum cultivars (Kegler and Hartmann, 1998; Waterworth and Hadidi, 1998; Hadidi and Candresse, 1999, 2001).

Currently, six PPV strains (types or subgroups) are recognized on the basis of serological and nucleic acid-based typing procedures. These are: PPV-Dideron (D), PPV-Marcus (M), PPV-Cherry (C), PPV-El Amar (EA), PPV-Winona (W), and PPV-Recombinant (Rec) (Candresse and Cambra, 2006). The first four strains are the most common and well studied. PPV-W

* Corresponding author. Tel.: +39 06 8207 0244; fax: +39 06 8207 0243.
E-mail address: m.barba@ispave.it (M. Barba).
[1] Lead Scientist Emeritus.

0166-0934/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jviromet.2007.08.019

was reported and eradicated from two infected plum trees in Canada (James et al., 2003). PPV-Rec is a natural recombinant between PPV-D and PPV-M strains (Glasa et al., 2004).

Some PPV strains may be distinguished by their biological properties. For example PPV-M is more damaging to infected peach plants than other strains. PPV-C infects cherry trees as well as other stone fruit trees; however, cherry trees are immune to infection by the other PPV strains. The identification of PPV in the infected samples as well as the identification of the specific PPV viral strain(s) is an important prerequisite for sharka control, especially in those countries in which the virus is endemic and the eradication of the disease is more difficult.

PPV is a very important quarantine agent that needs to be controlled by official phytosanitary measures aimed at preventing its entry and spread into countries where it does not occur (Barba, 1998; Foster and Hadidi, 1998). It is also a significant certification agent in many countries where it occurs so that its spread in stone fruit orchards may be controlled (Barba, 1998). Hence, the development and improvement of innovative technologies for PPV detection and differentiation of its strains are common objectives.

Historically, PPV has been detected by biological indexing on GF 305 peach indicator seedlings that produce typical PPV leaf shoe string symptoms in 4–6 weeks in the greenhouse (Gentit, 2006), and by enzyme-linked immunosorbent assay (ELISA) with polyclonal antibodies (Clark and Adams, 1977). The emergence of reverse transcription-polymerase chain reaction (RT-PCR) for plant pathogen detection (Hadidi and Yang, 1990) revolutionized plant viral diagnostics and made it possible to develop RT-PCR methods for PPV detection in the early 1990s (Wetzel et al., 1991, 1992; Levy and Hadidi, 1994).

Currently, PPV is detected by ELISA with polyclonal or monoclonal antibodies (Cambra et al., 2006), RT-PCR (Olmos et al., 2006), and quantitative real time RT-PCR (Schneider et al., 2004; Varga and James, 2005). The later technique detects the virus or its strains and provides quantification of the PPV viral target.

There are limitations to the current PPV detection methods. Biological indexing is time consuming, labor intensive and requires greenhouse space. ELISA depends on the quality and availability of PPV polyclonal and monoclonal antibodies; there are also several disadvantages for the use of polyclonal antibodies (Cambra et al., 2006).

Limitations on the use of monoclonal antibodies may include: (i) unsuccessful detection of PPV during the dormant period of stone fruit trees; (ii) assay results must be confirmed in key cases by molecular methods (Cambra et al., 2006); (iii) in some cases, typing of PPV isolates by subgroup-specific monoclonal antibodies does not always correspond with molecular typing, which may result in discrepancies (Candresse et al., 1998a; Quiot-Douine et al., 2001). In addition, the evolution of viral serotypes may interfere with the reliability of the method, and only one virus is detected per assay.

With RT-PCR and real-time RT-PCR methods, because of the difficulty in designing compatible multiplex primer sets, the maximum number of viruses or their strains detectable in a single assay is relatively small, and discrimination among viral strains may require additional labor-intensive procedures.

DNA microarrays were first described in 1995 (Schena et al., 1995) for simultaneous analysis of large-scale gene expression patterns. Since then, this technology has attracted great interest among biologists; recently it has been utilized in plant virus and other plant pathogen detection (for review see Hadidi et al., 2004; Barba and Hadidi, 2007). To address the limitations relating to the detection of PPV and its strains, a genomic approach, based on the viral nucleotide sequence has been developed. This approach uses oligonucleotide DNA microarray technology. More specifically, a long 70-mer oligonucleotide DNA microarray has been designed with the potential to simultaneously detect PPV and its strains from many samples in a single assay. This study describes the validation of this DNA microarray by using different strains of PPV from infected stone fruit or *Nicotiana benthamiana* leaves.

2. Materials and methods

2.1. Virus strains and their sources

The four common and well-studied strains of PPV (D, M, C and EA) were used in this investigation and maintained in plants that showed PPV symptoms in a greenhouse under controlled conditions. PPV-D in infected peach plants (ISPaVe 158.2) (the abbreviation ISPaVe stands for Istituto Sperimentale per la Patologia Vegetale) was from Rome, Italy. PPV-M in infected peach plants (ISPaVe 167.4) was from Veneto, Italy. PPV-C in infected *N. benthamiana* (ISPaVe 148.2) was obtained from Agritest company, Bari, Italy. PPV-EA in infected apricot plants (ISPaVe 172.3) was from Egypt.

N. glutinosa plants systemically infected with an isolate of *Turnip mosaic virus* (TuMV) (from Rome, Italy) and noninfected peach seedlings were used in control experiments. TuMV was chosen since it belongs to the genus *Potyvirus*, as does PPV. All members of this genus are characterized by several highly conserved genomic regions.

2.2. RNA extraction

Target RNA was extracted from the symptomatic leaves of plants infected by each of the four PPV strains as well as from plants infected with TuMV and uninfected plants. Total RNA was extracted from 100 mg of leaf tissue using a RNeasy Plant mini kit (Qiagen Inc., Valencia, CA). Total RNA (TRNA, host and viral) was recovered (50 μl) and its concentration was estimated by measuring its absorbance at 260 nm using a NanoDrop spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). A cDNA was obtained from plant TRNAs by using oligodT and random primers and its concentration was determined as above (see Section 2.5 for details).

2.3. Primer design for confirming the identity of PPV strains by cloning and sequencing

PPV strains used in this investigation were typed by the sequencing of clones obtained from RT-PCR products amplified using universal primers designed on the basis of the sequence alignment of the PPV strains. The nucleotide sequences of the primers are:

PPVUr: 5′-CCATACCCTGGCATGTATGC-3′ (reverse-sense); PPVMf: 5′- GAAGCATCTGAGACGGAAATTGAG-3′ (sense). This primer pair gave an amplified product of about 840 bp of the viral strain coat protein.

2.4. Microarray design and construction

The nucleotide sequences of 11 isolates of PPV were retrieved from NCBI GenBank: members of PPV-D strain (X16415, AY912056, DQ465242, AF401296, D13751); members of PPV-M strain (AJ243957, M92280); a member of PPV-EA strain (AM157175); PPV-SwC (Y09851) and PPV-SoC (X97398), members of the PPV-C strain; a member of PPV-W strain (AY912055). Other sequences of PPV isolates were obtained from published reports. The sequences were used to design specific 70-mer oligonucleotides for each isolate that were analyzed with EXIQON "Design oligonucleotides for expression arrays" software (http://oligo.lnatools.com/expression/).

The 70-mer oligonucleotides were designed on the basis of the alignment of the RNA genome of PPV strains and their isolates. Homology between individual 70-nt viral fragments and each strain genome of PPV species was assessed by the nucleotide identity score after BLASTN alignment. 70-mer oligonucleotides were designed to identify and genotype individual PPV strains. Other 70-mer oligonucleotides were designed to identify and genotype two or three PPV strains. A universal probe (named V-2) aimed at detecting all the PPV strains was designed from the most conserved PPV sequences, which is located in the 3′-untranslated region (UTR). The sequence of each oligonucleotide was blasted against the known nucleotide sequences of PPV isolates and other pathogens and showed no significant homology to other pathogens or other PPV strains, except the sequence of the oligonucleotide designed to detect two or more PPV strains that showed homology between the specific strains designed to detect (percentage of identity >85.0). Only those 70-mer oligonucleotide probes that do not self anneal (analyzed using the DNAMAN software, Lynnon Biosoft, Montreal, Canada) were retained (Table 1).

All oligonucleotides were synthesized with a 5′-amino modification (Syntezza Bioscience Ltd., Jerusalem, Israel) and UltraGAPS Microarray Slides (Corning, NY, USA) were printed at the Medical School of the Hebrew University, Jerusalem. Each oligonucleotide was suspended at a concentration of 10 μM in 3× SSC (1× SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0). The microarray design was as follows: each subarray contained six rows of ten spots; four subarrays were printed in each subarray row; the chip contained four subarray rows (total of 16 subarrays). The diameter of each spot was 150 μm and the space between two spots was 305 μm. Printing was done at 22 °C and 40% relative humidity.

Each PPV isolate-specific oligonucleotide was printed in 12 replicated spots on subarrays (Table 2). Two random sequence oligonucleotides were printed as negative control:

Oligo-1: 5′-AGTCGTCCAGTGCAGCTAGTTGCAGTCGTAAAGTCGGAATTGCGTGTGAGCGTACGTTGAGGTTGAAAC-3′; Oligo-2: 5′-GATTGTGTTTAGACACACACGCTATATGCGCGCTATATAGCCCGGGTTTTTGCTCGTAGTCTTTAGG-3′.

Table 1
70-mer oligonucleotide probes used for the detection and genotyping of PPV and its strains

PPV		Selected 70-mer oligonucleotides		
Isolate[a]	Strain	Position on the sequence of PPV genome	Position on PPV genes	% identity to PPV strain
D-CP	D	8656–8726	CP	D (100)
F-2	D	8643–8713	CP	D (98)
P-2	D	2940–3010	P3	D (100)
M-1	M	7769–7839	NIb	M (100)
M-CP	M	8653–8722	CP	M (100)
SoC-1	C	403–473	P1	C (100)
SoC-2	C	1657–1727	HCpro	C (98)
SwC-1	C	7408–7478	NIb	C (98)
SwC-2	C	3099–3169	P3	C (98)
EA-CP	EA	8659–8729	CP	EA (100)
W-1	W	7403–7473	NIb	W (100)
W-3	W	5344–5414	CI	W (100)
F-3	D	2032–2102	HCpro	D (100), M (100)
EA-1	EA	5330–5400	CI	EA (100), D (88)
D-3	D	9565–9635	CP-3′-UTR	D (97), M (90), C (90)
V-2[b]	D	9609–9679	3′-UTR	D (95), M (91), C (91), EA (90)
V-3	D	2855–2925	P3	D (100)
Oligo-1	–	–	–	None
Oligo-2	–	–	–	None

Oligo-1 and oligo-2 are 70-mer oligonucleotides with random sequences used as negative controls.
[a] Abbreviations of PPV isolates: EA, El Amar (PPV-EA); W, Winona (PPV-W); F, Fantasia (PPV-D); SoC, Sour cherry (PPV-C); M, Marcus (PPV-M); P, Pennsylvania (PPV-D); SwC, Sweet cherry (PPV-C); V, Vulcan (PPV-D).
[b] Considered as a universal probe.

Table 2
Representation of the spot position of each 70-mer oligonucleotide probe in the array

70-mer oligonucleotide probe[a]	Spot position[b]											
PPV-EA-1	IA1	IC1	IE1	IB5	ID5	IF5	IA6	IC6	IE6	IB10	ID10	IF10
PPV-W-3	IB1	ID1	IF1	IA5	IC5	IE5	IB6	ID6	IF6	IA10	IC10	IE10
PPV-EA-CP	IA2	IC2	IE2	IB4	ID4	IF4	IA7	IC7	IE7	IB9	ID9	IF9
PPV-F-2	IB2	ID2	IF2	IA4	IC4	IE4	IB7	ID7	IF7	IA9	IC9	IE9
PPV-F-3	IA3	IB3	IC3	ID3	IE3	IF3	IA8	IB8	IC8	ID8	IE8	IF8
PPV-W-1	IIA1	IIC1	IIE1	IIB5	IID5	IIF5	IIA6	IIC6	IIE6	IIB10	IID10	IIF10
PPV-SoC-1	IIB1	IID1	IIF1	IIA5	IIC5	IIE5	IIB6	IID6	IIF6	IIA10	IIC10	IIE10
PPV-M-CP	IIA2	IIC2	IIE2	IIB4	IID4	IIF4	IIA7	IIC7	IIE7	IIB9	IID9	IIF9
PPV-SoC-2	IIB2	IID2	IIF2	IIA4	IIC4	IIE4	IIB7	IID7	IIF7	IIA9	IIC9	IIE9
PPV-M-1	IIA3	IIB3	IIC3	IID3	IIE3	IIF3	IIA8	IIB8	IIC8	IID8	IIE8	IIF8
PPV-P-2	IIIA1	IIIC1	IIIE1	IIIB5	IIID5	IIIF5	IIIA6	IIIC6	IIIE6	IIIB10	IIID10	IIIF10
PPV-D-CP	IIIB1	IIID1	IIIF1	IIIA5	IIIC5	IIIE5	IIIB6	IIID6	IIIF6	IIIA10	IIIC10	IIIE10
PPV-3	IIIA2	IIIC2	IIIE2	IIIB4	IIID4	IIIF4	IIIA7	IIIC7	IIIE7	IIIB9	IIID9	IIIF9
PPV-SwC-1	IIIB2	IIID2	IIIF2	IIIA4	IIIC4	IIIE4	IIIB7	IIID7	IIIF7	IIIA9	IIIC9	IIIE9
PPV-SwC-2	IIIA3	IIIB3	IIIC3	IIID3	IIIE3	IIIF3	IIIA8	IIIB8	IIIC8	IIID8	IIIE8	IIIF8
PPV-V-2	IVA1	IVC1	IVE1	IVB5	IVD5	IVF5	IVA6	IVC6	IVE6	IVB10	IVD10	IVF10
PPV-V-3	IVA2	IVC2	IVE2	IVB4	IVD4	IVF4	IVA7	IVC7	IVE7	IVB9	IVD9	IVF9
Random oligo-1	IVB2	IVD2	IVF2	IVA4	IVC4	IVE4	IVB7	IVD7	IVF7	IVA9	IVC9	IVE9
Random oligo-2	IVA3	IVB3	IVC3	IVD3	IVE3	IVF3	IVA8	IVB8	IVC8	IVD8	IVE8	IVF8

[a] The probes were designed from the nucleotide sequences of PPV isolates (see Table 1). Abbreviations of PPV isolates: EA, El Amar (PPV-EA); W, Winona (PPV-W); F, Fantasia (PPV-D); SoC, Sour cherry (PPV-C); M, Marcus (PPV-M); P, Pennsylvania (PPV-D); SwC, Sweet cherry (PPV-C); V, Vulcan (PPV-D).

[b] For example, the 70-mer oligonucleotide PPV-EA-1 was spotted 12 times in subarray I: in line 1 of rows A, C, and E; in line 5 of rows B, D, and F; in line 6 of rows A, C, and E; in line 10 of rows B, D, and F (see Fig. 2).

2.5. cDNA synthesis of total plant RNA

The reverse transcription (RT) reactions (final volume of 30 μl) were prepared with 2–5 μg of total RNA, 2 μM random hexamers and 6 mM oligodT. The mixture was heated for 10 min at 70 °C and immediately cooled on ice. To the mixture were added 5 mM dithiothreitol, dNTPs/aa-dUTP (a mixture of 10 mM dGTP, dATP, dCTP each, 2 mM dTTP, and 8 mM aminoallyl-dUTP), 400 units of Superscript III reverse transcriptase with first-strand buffer (Invitrogen Corp., Carlsbad, CA). The mixture was incubated for 3 h at 50 °C, followed by 15 min at 70 °C, and finally for 30 min at 4 °C. The cDNA reaction was incubated with 30 μg RNase A and 2× SSC for 30 min at 37 °C (final volume of 50 μl). The cDNA was cleaned using a Microcon YM-30 column (Millipore Corporation, Bedford, MA, USA) dried and suspended in 8 μl of nuclease-free water and labeled as described below.

2.6. Fluorescent labeling of cDNA with cyanine 3 or cyanine 5

The indirect chemical labeling of cDNA was done according to the procedure described in the URL (with modifications) http://schnablelab.plantgenomics.iastate.edu/docs/resources/protocols/pdf/cDNA_microarray.2007.04.01.pdf. Eight microlitres of cDNA were added to 1 μl of $Na(HCO_3)_2$ buffer pH 9.0 and 1 μl of Cy3 or Cy5 dye (Amersham Bioscience, Buckinghamshire, UK) suspended in DMSO buffer and incubated for 18 h at room temperature. The labeled cDNA was ethanol precipitated and purified using a QiaQuick PCR cleaning kit (Qiagen). Labeling efficiency was monitored by running an aliquot of the Cy3 or Cy5 labeled cDNA on an agarose gel together with fluorochromes quantitative standards and reading fluorescence with a Typhoon 9200 (Amersham) fluorescence reader.

Dye-swapping was performed on the PPV strains PPV-D and PPV-M to balance any interference due to the fluorophore by repeating the experiment in different slides (Churchill, 2002; Yang and Speed, 2002).

2.7. Hybridization

Slides were pre-hybridized for 45 min at 55 °C with a pre-heated (55 °C) blocking buffer (1% BSA, 0.1% SDS, 5× SSC). The slides were then washed with water and dried by centrifugation for 2 min at 2000 g. The hybridization buffer contained 1 μg of each purified labeled cDNA (typically 30 pmol of incorporated dye), 50% formamide, 0.1% SDS, 5× SSC was denatured for 3 min at 95 °C, cooled on ice for 30 s and briefly spun down. The buffer was applied to the slide and covered with a cover slip (HybriSlip, Schleicher and Schuell Bioscience, Keene, NH). Hybridization was performed for 20 h at 55 °C in the dark. The slides were then washed with pre-heated 2× SSC, 0.1% SDS at 55 °C (5 min, twice), followed by 0.5× SSC, 0.1% SDS (10 min at an ambient temperature) and finally with 0.05× SSC (5 min at an ambient temperature, four times).

2.8. Scanning

The hybridized slides were scanned using a GenePix 4200A array scanner (Axon Instruments Ltd., Aberdeen, Scotland, UK) containing a 532 nm laser for Cy3 and a 635 nm laser for Cy5 fluorescence measurement. Laser power was fixed at 70% of its potentiality for both lasers, while photomultiplier tube (PMT) power ranged from 45 to 65%, depending on signal intensity. The level of PMT power was selected to balance the background with the level of spots detection on a slide. Fluorescent intensities were quantified by using GenePix Pro 3.0 Software (Axon Instruments Ltd.). The DNA spots were then identified on the array and located using a Gal file as feature indicator, such that measurements of fluorescence and local background fluorescence for each DNA spot could be recorded. The parameters 'mean signal–mean local background' (mean Cy3 minus B or mean Cy5 minus B) and the 'mean local background' (B) were used in further calculations. Local background was calculated using the adaptive circle method. Signals were considered positive if at least fivefold above the local background.

3. Results

3.1. Identification of PPV isolates used for hybridization following cloning and sequencing of the coat protein gene

Cloning and sequencing of the coat protein gene of the four PPV isolates, ISPaVe 158.2, ISPaVe 167.4, ISPaVe 172.3, and ISPaVe 148.2 confirmed that they belonged to PPV-D, PPV-M, PPV-EA, and PPV-C strains, respectively. Total RNAs extracted from leaves of plants infected with each of the PPV strain were used for cDNA synthesis, labeling and hybridization.

3.2. Total RNA extraction and cDNA synthesis

On average, 100 mg of tissue yielded about 2.5 μg total RNA. cDNA synthesis using Superscript III RT enzyme was very efficient, as the cDNA yield was practically equivalent to the total RNA input, confirming that aminoallyl-dUTP incorporation did not interfere with the efficiency of cDNA synthesis.

3.3. Design of 70-mer oligonucleotide probes

Each of the designed selected 70-mer oligonucleotide probes (Table 1) hybridized to its specific target except one, M-CP. This could be due to problems encountered during the printing process, unexpected hairpin structures and/or incorrect nucleotide sequences.

The percentage of identity that each oligonucleotide probe shared with its target was significantly high (Table 1), which resulted in specific hybridization of the probe to its target. Moreover, no cross hybridization was observed below 85% identity. Fig. 1 shows the alignment of one of the selected probes, PPV-D-specific PPV-DCP, against PPV strains and PPV-D isolates. The significantly high identity between the probe and its target is clearly shown.

3.4. Detection and genotyping of PPV strains

The initial microarray hybridization experiments were performed by using Cy5-labeled cDNA from PPV-D infected peach (ISPaVe 158.2) together with Cy3-labeled cDNA of non-infected peach seedlings (Fig. 2, panel A). The cDNA was synthesized from 5 μg of total RNA. Further experiments showed that 2.0 μg of total RNA could be used without a significant loss of signal (data not shown). Hence, 2.0 μg of total RNA were used for cDNA synthesis for subsequent hybridizations (Fig. 2, panels B–D).

Hybridizations were dependent on PPV infection. Hybridization with a mixture of cDNAs from PPV-D infected (Cy5-labeled) and non-infected (Cy3-labeled) peach leaves produced only a PPV-related Cy5 signal (Fig. 2, panel A). Similarly only the Cy5 PPV-related signals were obtained when a mixture of cDNAs from PPV-M infected (Cy5-labeled) and TuMV-infected (Cy3-labeled) plant samples were used (Fig. 2, panel B).

To test the virus strain specificity of the probes present on the microarray, the hybridization patterns of cDNA mixtures obtained from plants infected with different PPV strains were compared (Fig. 2, panels C and D). In the results shown in Fig. 2, panels C and D; hybridizations were performed with mixtures of cDNAs from two different PPV strains. In panel C, the microarray was hybridized with a mixture of cDNAs from plants infected with PPV-C (Cy5) and PPV-M (Cy3). In panel D, the PPV microarray was hybridized with a mixture of cDNAs from plants infected with PPV-EA (Cy5) and PPV-D (Cy3). For clarity, each scan is shown separately.

Each virus strain provided a specific hybridization pattern. As it is evident from the Cy3 fluorescence images of panels C and D, the pattern obtained in conditions of competition with another PPV strain is remarkably similar to the one obtained in double hybridization using cDNA from uninfected plants (panel A) or from TuMV-infected plants (panel B), and it is reproducible after dye-swapping, including that of PPV-D (panels A and D) and PPV-M (panels B and C). This observation suggests that multiplex use of this microarray chip may be possible.

Table 3 summarizes the results of the hybridizations and defines the strain-specificity of the probes. Seventeen PPV 70-mer oligonucleotide probes and two random 70-mer oligonucleotide probes were tested for their ability to detect and genotype PPV strains. The two random oligonucleotides did not hybridize to any of the labeled cDNAs, demonstrating that they were correctly designed to serve as negative controls.

Similarly, the PPV universal probe V-2 corresponding to the most conserved PPV sequence (3′-UTR) hybridized with the four strains tested: D, M, C and EA. Four probes (D-CP, F-2, P-2, V-3) specifically hybridized to PPV-D cDNA. Another four probes (SoC-1, SoC-2, SwC-1, SwC-2) specifically hybridized to PPV-C cDNA. One probe (M-1) hybridized specifically to PPV-M isolate. One probe (EA-CP) hybridized to PPV-EA only. Two probes (F-3, EA-1) hybridized with two PPV strains (each probe with different strains). The PPV-3 probe hybridized with three PPV strains (all except PPV-EA). Two probes for PPV-W (W-1 and W-3) were not tested because of the unavailability of PPV-W infected tissue. These probes, however, did not hybridize to PPV-D, PPV-M, PPV-C or EA (Fig. 2 and Table 3), which may suggest their specificity to the PPV-W strain.

```
(A)
Sequence format is Pearson
Sequence 1: D-CP              70 bp
Sequence 2: PPVD-X16415     8750 bp
Sequence 3: PPVM-AJ243957   9786 bp
Sequence 4: PPVSwC-Y09851   9795 bp
Sequence 5: PPVEA-AM157175  9791 bp
Sequence 6: PPVW-AY912055   9788 bp
Start of Pairwise alignments
Aligning...
Sequences (1:2) Aligned. Score:  100
Sequences (1:3) Aligned. Score:  77
Sequences (1:4) Aligned. Score:  64
Sequences (1:5) Aligned. Score:  65
Sequences (1:6) Aligned. Score:  57

PPVSwC           CCACAGCAGTTTCCACGCCTGCAGTAACAAGCTCACAATTTCCACCTCCACCACTCCCAA 8697
PPVW             TAGCAACAACACCTCCATCCACCGCAACGGCACCACAGGTAACAGCCACATCGACCCAGC 8691
PPVEA            CCACTACACAGCAGCCAATTGTTACCACAACAACTCAGCAAACTCCAATAACGAGTGCAA 8691
D-CP             ------------------------CAATACTTCAACCACCTCCAGTCATACAGCCTGCAC 36
PPV-DX16415      TTACTGCACCGGCAGCAACTAGCCCAATACTTCAACCACCTCCAGTCATACAGCCTGCAC 8691
PPVM             TAACTGCACCGGCAGCAACTGTGGCAACGACTCAACCAGCTCCAGTGATACAACCTGCAC 8688
                                           *        *      *      *

PPVSwC           ATCTGCAGAGCACGGCACCAATGTTTGATCCCATATTCACTCCAGCAACAACCCAGCCAA 8757
PPVW             CCCTGCAGAGCACAACGTCAATGTTCAACCCCGTCTTTACACCAGCAACAACTCAACCGA 8751
PPVEA            CCTTACAAGCTACGCAGGCAATGTTTAATCCCATCTTCACTTCAGCGACGACTGAGCCGG 8751
D-CP             CC---CGGACTACGGCGCCAATGCTCAACCCCATTTT----------------------- 70
PPV-DX16415      CC---CGGACTACGGCGCCAATGCTCAACCCCATTTTCACGCCAGCAACAACTCAACCAG 8748
PPVM             CC---CAAACCACAGCACCAATGTTCAACCCCATTTTCACTCCAGCAACAACTCAGCCTG 8745
                      *     **     ***** *  * *** * **

(B)
Sequence format is Pearson
Sequence 1: D-CP                      70 bp
Sequence 2: PPVD-AF401296            9786 bp
Sequence 3: PPVD-D13751             9741 bp
Sequence 4: PPVD-AY912056           9786 bp
Sequence 5: PPVD-DQ465242           9786 bp
Sequence 6: PPVD-X16415             9787 bp
Start of Pairwise alignments
Aligning...
Sequences (1:2) Aligned. Score:  100
Sequences (1:3) Aligned. Score:  94
Sequences (1:4) Aligned. Score:  98
Sequences (1:5) Aligned. Score:  98
Sequences (1:6) Aligned. Score:  100

PPVD                    --------------------CTTCAACCACCTCCAGTCATACAGCCTGCACCCCGGACTA 8652
PPVDFantasiaAY912056    CGGCAGCAACTAGCCCAATACTTCAACCACCTCCAGTCATACAGCCTGCACCCCGGACTA 8697
D-CP                    ---------------CAATACTTCAACCACCTCCAGTCATACAGCCTGCACCCCGGACTA 45
PPV-DX16415             CGGCAGCAACTAGCCCAATACTTCAACCACCTCCAGTCATACAGCCTGCACCCCGGACTA 8700
PPDVPenn2AF401296       CGGCAGCAACTAGCCCAATACTTCAACCACCTCCAGTCATACAGCCTGCACCCCGGACTA 8697
PPVDPenn3DQ465242       CGGCAGCAACTAGCCCAATACTTCAACCACCTCCAGTCATACAGACTGCACCCCGGACTA 8697
                                            ************************ ***************

PPVD                    CGGCGCCAATGCTCAACCCCATTTTCACGCCAGCAACAACTCAACCAGCAACAAAACCAG 8712
PPVDFantasiaAY912056    CGGCGCCAATGTTCAACCCCATTTTCACGCCAGCAACAACTCAACCAGCAACAAAACCAG 8757
D-CP                    CGGCGCCAATGCTCAACCCCATTTT----------------------------------- 70
PPV-DX16415             CGGCGCCAATGCTCAACCCCATTTTCACGCCAGCAACAACTCAACCAGCAACAAAACCAG 8760
PPDVPenn2AF401296       CGGCGCCAATGCTCAACCCCATTTTCACGCCAGCAACAACTCAACCAGCAACAAGACCAG 8757
PPVDPenn3DQ465242       CGGCGCCAATGCTCAACCCCATTTTCACGCCAGCAACAACTCAACCAGCAACAAGACCAG 8757
                        *********** *************
```

Fig. 1. Alignment of the PPV-D-specific 70-mer oligonucleotide probe PPV-CP against the nucleotide sequences of PPV strains (A) or PPV-D isolates (B) retrieved from GenBank. The alignment score of each PPV strain and PPV-D isolate is also shown.

4. Discussion

In this study a PPV detection and genotyping method, based on a combination of viral genomics and long oligonucleotide DNA microarray technology, was developed. The high-performance characteristics of long oligonucleotide microarrays has been documented (Hughes et al., 2001; Wang et al., 2002). To apply this technology, 70-mer oligonucleotides were designed to allow the entire spectrum of PPV strains to be detected. Other oligonucleotides were designed to enable detection of single strains or a mixture of two or more strains. Plants infected by one strain or by multiple strains (mixed infection) can be detected.

Levy and Hadidi (1994) demonstrated that the 3′-UTR of the PPV genome is highly conserved in all PPV strains. Thus, a stretch of 70 nucleotides from this region was selected for microarray-based detection of all known strains of the virus. By using the most conserved sequences in individual and all PPV strains, the probability that all known and unknown strains of PPV could be detected simultaneously using the microarray was maximized. This could be achieved because of the high resolution of microarray hybridization to differentiate among PPV strains.

In a significant number of microarray experiments, RNA standards were used that contain an RNA sample from a particular

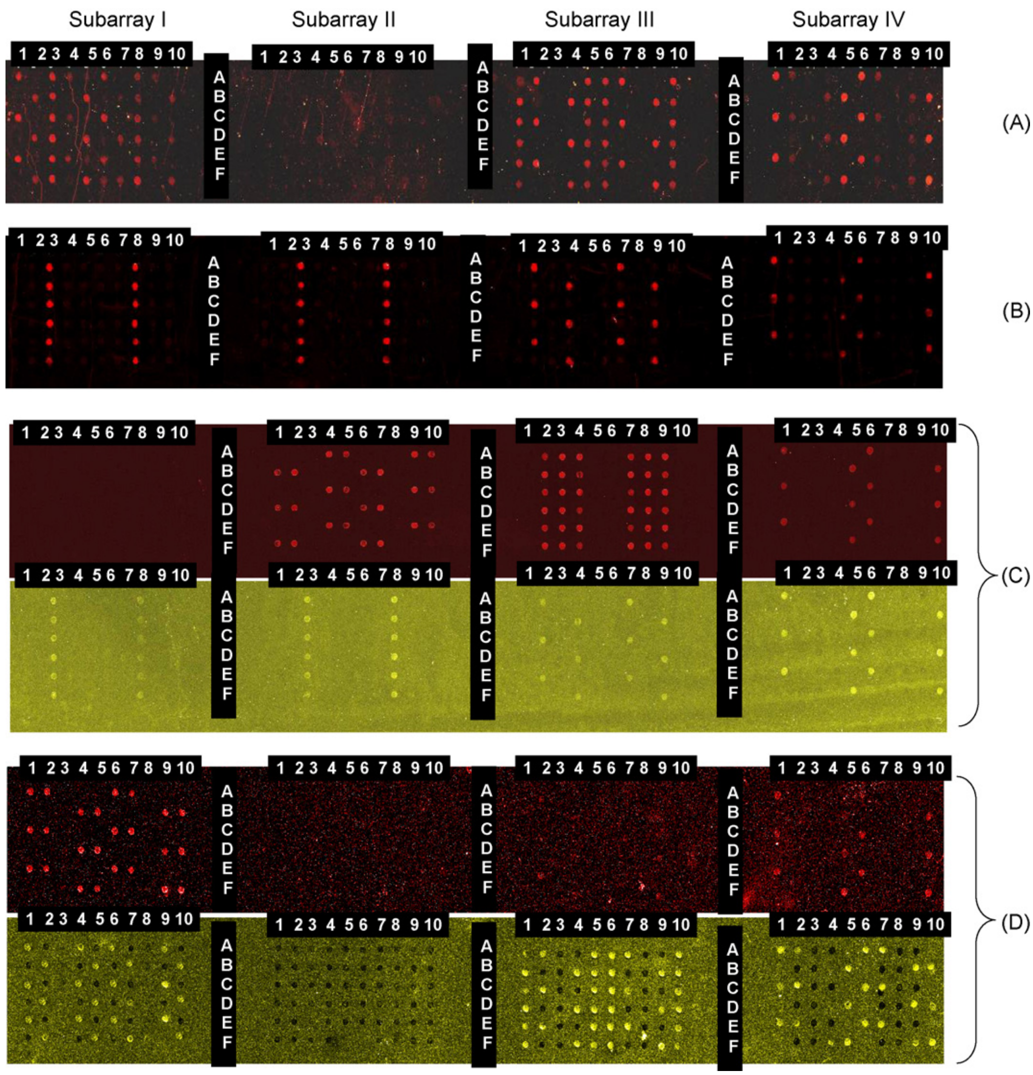

Fig. 2. Detection of PPV strains using the designed 70-mer oligonucleotides PPV microarrays. (Panel A): Hybridization with cDNA of PPV-D infected sample (Cy5-labeled) and cDNA of non-infected peach (Cy3-labeled). (Panel B): cDNA of PPV-M infected sample (Cy5-labeled) and cDNA of TuMV-infected sample (Cy3-labeled). (Panel C): cDNA of PPV-C infected sample (Cy5-labeled) and cDNA of PPV-M infected sample (Cy3-labeled). (Panel D): cDNA of PPV-EA infected sample (Cy5-labeled) and cDNA of PPV-D infected sample (Cy3-labeled). For panels (A) and (B) only the composite image is shown. For panels (C) and (D), the upper panel shows hybridization with the Cy5-labeled cDNA, and the lower panel with the Cy3-labeled cDNA. The Cy3 and Cy5 composite images are not shown for clarity and to facilitate hybridization analysis. The spot position of each specific 70-mer oligonucleotide probe in the array is reported in Table 2.

time point, tissue or a set of reference genes (Iyer et al., 1999; Manduchi et al., 2000; Stingley et al., 2000). RNA standards could be problematic and unreliable due to their variability in relative amounts and their instability (Talaat et al., 2000). Furthermore, using host mRNAs as a reference standard requires that their expression is invariant during virus replication. Fluorescent dye-labeled genomic DNA was found to be a more reliable co-hybridization standard for gene expression analyses in *Mycobacterium tuberculosis* and in mouse (Talaat et al., 2002; Williams et al., 2004, 2006), and very recently in the insect baculovirus *Choristoneura fumiferena* nucleopolyhedrovirus (Yang et al., 2007). In the present investigation, the use of fluorescent dye-labeled PPVcDNA was also found very efficient and reliable co-hybridization standard for PPV detection and genotyping analyses, thus, the need of using RNA standards was eliminated.

In a first set of experiments (Fig. 2, panels A and B), the hybridization signal of Cy5-labeled cDNA from peach leaves infected with PPV was compared with that of Cy3-labeled cDNA from uninfected or TuMV-infected leaves. These experiments demonstrated several superior features of PPV strain detection using DNA microarrays. The PPV signature was readily detected and identified by specific hybridization to the appropriate oligonucleotides. cDNA from non-infected peach leaves did not hybridize to any oligonucleotides. Furthermore, dye-swapping experiments (Fig. 2, panels C and D) minimized any bias that may come from the systematic differences between green and red intensities of the Cy3 and Cy5 dyes respectively and confirmed the reliability of results. The indirect labeling method with Cy dyes coupling after cDNA synthesis enhanced the detection sensitivity.

Table 3
70-mer oligonucleotides hybridizing with each PPV strain

70-mer Oligonucleotide Probes	PPV strains - targets			
	D	M	C	EA
PPV-D-CP (D)	■			
PPV-F-2 (D)	■			
PPV-F-3 (D)	■	■		
PPV-M-1 (M)		■		
PPV-M-CP (M)				
PPV-SoC-1 (C)			■	
PPV-SoC-2 (C)			■	
PPV-SwC-1 (C)			■	
PPV-SwC-2 (C)			■	
PPV-EA-CP (EA)				■
PPV-EA-1 (EA)	■			■
PPV-W-1 (W)				
PPV-W-3 (W)				
PPV-P-2 (D)	■			
PPV-V-3 (D)	■			
PPV-V-2 (D)	■	■	■	■
PPV-3 (D)	■	■	■	
random oligo- 1				
random oligo- 2				

Grey rectangles indicate positive hybridization to individual strains (D, M, C, and EA). Results were derived from microarray hybridizations (Fig. 2).

In previous reports aimed at detecting plant pathogens using microarrays, labeling was obtained by incorporation of Cy-labeled nucleotides during a PCR step (Call et al., 2003). Alternatively, in some cases (Boonham et al., 2003; Lee et al., 2003; Abdullahi et al., 2005), labeling was performed during the reverse transcription step of total RNA with Cy-labeled nucleotides. This usually resulted in a decrease in the amount of cDNA obtained, possibly because of steric constrains imposed by the Cy modification.

Only recently a report proposed a method for plant virus detection that couples the use to oligonucleotide microarrays with indirect fluorochrome labeling (Bystricka et al., 2005). The procedure used in the present study eliminated the PCR amplification step that is usually performed to increase the sensitivity of this technology. High sensitivity is crucial for PPV diagnosis as it is found at a low titer in infected tissue and it is irregularly distributed in infected stone fruit trees. By eliminating the PCR step, the identification of PPV and its strains is limited only by the spectrum of the virus and strain probes present on the microarray. This is in contrast to PPV RT-PCR detection methods, which are restricted by the initial selection of targets and corresponding primers. Even when degenerate RT-PCR is used, the range of target strains may be narrow as an unambiguous identification may require additional labor intensive procedures such as restriction enzyme analysis, sequencing or hybridization of the PCR products (Candesse et al., 1998b; Vinje and Koopmans, 2000; Liolios et al., 2001; Wang et al., 2002). A DNA microarray composed of carefully selected PPV strain sequences bypasses these limitations and yields an extremely broad-reaching and unbiased detection strategy.

An oligonucleotide DNA microarray diagnostic tool for PPV and its strains as described in this study has many uses, such as in PPV certification and quarantine programs, studying PPV epidemiology in stone fruit orchards, insect transmission of the virus and its host range and should be of a great help in facilitating the discovery of new PPV strains. An oligonucleotide DNA microarray containing the complete PPV genome sequence may be used for studying PPV–host gene interaction, especially those of thale cress *Arabidopsis thaliana* whose genome size is 130 Mb and its complete nucleotide sequence has been published and/or peach *Prunus persica* whose genome size is 270 Mb and currently is being sequenced (Pennisi, 2007). These studies may lead to the development of plants resistant or tolerant to PPV infection. Thus PPV oligonucleotide DNA microarrays have the potential to be used in disease management and PPV control which have direct effect on security of the food supply.

Acknowledgement

This research was supported by a NATO funded project entitled "Microarray-Based Detection of Plant Viruses and Virus-Like Pathogens", Project No. LST. MD. SFP981023.

References

Abdullahi, I., Koerbler, M., Stachewicz, H., Winter, S., 2005. The 18S rDNA sequence of Synchytrium endobioticum and its utility in microarrays for the simultaneous detection of fungal and viral pathogens of potato. Appl. Microbiol. Biotechnol. 68, 368–375.

Barba, M., 1998. Virus certification of fruit tree propagative material in Western Europe. In: Hadidi, A., Khaterpal, R.K., Koganezawa, H. (Eds.), Plant Virus Disease Control. APS Press, St. Paul, MN, USA, pp. 288–293.

Barba, M., Hadidi, A., 2007. DNA microarrays: technology, applications, and potential applications for the detection of plant viruses and virus-like pathogens. In: Rao, G.P., Valverdi, R.A., Dovas, C.I. (Eds.), Techniques in Diagnosis of Plant Viruses. Studium Press LLC, Houston, TX, pp. 227–247.

Boonham, N., Walsh, K., Smith, P., Madagan, K., Graham, I., Barjer, I., 2003. Detection of potato viruses using microarray technology: towards a generic method for plant viral disease diagnosis. J. Virol. Methods 108, 181–187.

Bystricka, D., Lenz, O., Mraz, I., Piherova, L., Kmoch, S., Sip, M., 2005. Oligonucleotide-based microarray: a new improvement in microarray detection of plant viruses. J. Virol. Methods 128, 176–182.

Call, D.R., Boruki, M.K., Loge, F.J., 2003. Detection of bacterial pathogens in environmental samples using DNA microarrays. J. Microbiol. Methods 53, 235–243.

Cambra, M., Boscia, D., Myrta, A., Palkovics, M., Navratil, M., Barba, M., Gorris, M.T., Capote, N., 2006. Detection and characterization of Plum pox virus: serological methods. EPPO Bull. 36, 254–261.

Candesse, T., Hammod, R.W., Hadidi, A., 1998b. Detection and identification of plant viruses and viroids using polymerase chain reaction. In: Hadidi, A., Khaterpal, R.K., Koganezawa, H. (Eds.), Plant Virus Disease Control. APS Press, St. Paul, MN, USA, pp. 399–416.

Candresse, T., Cambra, M., 2006. Causal agent of sharka disease: historical perspective and current status of Plum pox virus strains. EPPO Bull. 36, 239–246.

Candresse, T., Cambra, M., Dallot, S., Lanneau, M., Asensio, M., Gorris, M.T., Reverse, F., Macquaire, G., Olmos, A., Boscia, D., Quiot, J.B., Dunez, J., 1998a. Comparison of monoclonal antibodies and polymerase chain reaction

assays for the typing of isolates belonging to the D and M serotypes of plum pox potyvirus. Phytopathology 88, 198–204.
Churchill, G.A., 2002. Fundamentals of experimental design for cDNA microarrays. Nat. Genet. 32 (Suppl.), 490–495.
Clark, M.F., Adams, A.N., 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34, 475–483.
Foster, J.A., Hadidi, A., 1998. Exclusion of plant viruses. In: Hadidi, A., Khaterpal, R.K., Koganezawa, H. (Eds.), Plant Virus Disease Control. APS Press, St. Paul, MN, USA, pp. 208–229.
Gentit, P., 2006. Detection of plum pox virus: biological methods. EPPO Bull. 36, 251–253.
Glasa, M., Palkovics, L., Komínek, P., Labonne, G., Pittnerová, S., Kúdela, O., Candresse, T., Šubr, Z., 2004. Geographically and temporally distant natural recombinant isolates of plum pox virus (PPV) are genetically very similar and form a unique PPV subgroup. J. Gen. Virol. 85, 2671–2681.
Hadidi, A., Candresse, T., 1999. Data sheet on plum pox virus. Global Crop Prot. Compend..
Hadidi, A., Candresse, T., 2001. Plum pox. In: Maloy, O.C., Murray, T.D. (Eds.), Encyclopedia of Plant Pathology. John Wiley and Sons, New York, US, pp. 788–791.
Hadidi, A., Yang, X., 1990. Detection of pome fruit viroids by enzymatic cDNA amplification. J. Virol. Methods 30, 261–270.
Hadidi, A., Czosnek, H., Barba, M., 2004. DNA microarrays and their potential applications for the detection of plant viruses, viroids and phytoplasma. J. Plant Pathol. 86, 97–104.
Hughes, T.R., Mao, M., Jones, A.R., Burchard, J., Marton, M.J., Shannon, K.W., Lefkowitz, S.M., Ziman, M., Schelter, J.M., Meyer, M.R., Sumire Kobayashi, S., Davis, C., Dai, H., He, Y.D., Stephaniants, S.B., Cavet, G., Walker, W.L., West Coffey, A.E., Daniel, D., Shoemaker, D.D., Stoughton, R., Blanchard, A.P., Friend, S.H., Linsley, P.S., 2001. Expression profiling using microarrays fabricated by an ink-jet oligonucleotide synthesizer. Nat. Biotechnol. 19, 342–347.
Iyer, V.R., Eisen, M.B., Ross, D.T., Schuler, G., Moore, T., Lee, J.C., Trent, J.M., Stuadt, L.M., Hudson Jr., J., Boguski, M.S., Lashkari, D., Shalon, D., Botstein, D., Brown, P.O., 1999. The transcriptional program in the response of human fibroblasts to serum. Science 283, 83–87.
James, D., Varga, A., Thompson, D., Hayes, S., 2003. Detection of a new and unusual isolate of plum pox poty virus in plum (*Prunus domestica*). Plant Dis. 87, 1119–11124.
Kegler, H., Hartmann, W., 1998. Present status and controlling conventional strains of plum pox virus. In: Hadidi, A., Khaterpal, R.K., Koganezawa, H. (Eds.), Plant Virus Disease Control. APS Press, St. Paul, MN, USA, pp. 616–628.
Lee, G.P., Min, B.E., Kim, C.S., Choi, S.H., Harn, C.H., Kim, S.U., Ryu, K.H., 2003. Plant virus cDNA chip hybridization for detection and differentiation of four cucurbit-infecting Tobamoviruses. J. Virol. Methods 110, 19–24.
Levy, L., Hadidi, A., 1994. A simple and rapid method for processing tissue infected with plum pox potyvirus for use with specific 3′ non coding region RT-PCR assays. EPPO Bull. 24, 595–604.
Liolios, L., Jenney, A., Spelman, D., Kotsimbos, T., Catton, M., Wesselingh, S., 2001. Comparison of a multiplex reverse transcription-PCR-enzyme hybridization assay with conventional viral culture and immunofluorescence techniques for the detection of seven viral respiratory pathogens. J. Clin. Microbiol. 39, 2779–2783.
Manduchi, E., Grant, G.R., McKenzie, S.E., Overton, G.C., Surrey, S., Stoeckert, C.J., 2000. Generation of patterns from gene expression data by assigning confidence to differentially expressed genes. Bioinformatics 16, 685–698.
Olmos, A., Capote, N., Candresse, T., 2006. Detection and characterization of Plum pox virus: molecular methods. EPPO Bull. 36, 262–266.
Pennisi, E., 2007. The greening of plant genomics. Science 317, 317.
Quiot-Douine, L., Boeglin, M., Dallot, S., Marie-Jeanne, V., Quiot, J.B., 2001. *Plum pox virus* diversification observed in a small cluster of orchards. Acta Hortic. 550, 85–90.
Schena, M., Shalon, D., Davis, R.W., Brown, P.O., 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 270, 467–470.
Schneider, W.L., Sherman, D.J., Stone, A.L., Damsteeg, V.D., Frederick, R.D., 2004. Specific detection and quantification of Plum pox virus by real-time fluorescent reverse transcripton-PCR. J. Virol. Methods 120, 97–105.
Stingley, R.W., Ramirez, J.J., Aguilar, S.A., Simmen, K., Sandri-Goldin, R.M., Ghazal, P., Wagner, E.K., 2000. Global analysis of herps simplex virus type 1 transcription using an oligonucleotide-based DNA microarrays. J. Virol. 74, 9916–9927.
Talaat, A.M., Howard, S.T., Hale IV, W., Lyon, R., Garner, H., Johnston, S.A., 2002. Genomic DNA standards for gene expression profiling in Mycobacterium tuberculosis. Nucleic Acids Res. 30, e104.
Varga, A., James, D., 2005. Detection and differentiation of plum pox virus using real-time multiplex PCR with SYBR Green and melting curve analysis: a rapid method for strain typing. J. Virol. Methods 123, 213–220.
Vinje, J., Koopmans, M.P., 2000. Simultaneous detection and genotyping of “Norwalk-like” viruses by oligonucleotide array in a reverse line blot hybridization format. J. Clin. Microbiol. 38, 2595–2601.
Wang, D., Coscoy, L., Zylberberg, M., Avila, P.C., Boushey, H.A., Ganem, D., De Risi, J.L., 2002. Microarray-based detection and genotyping of viral pathogens. Proc. Natl. Acad. Sci. U.S.A. 99, 15687–15692.
Waterworth, H.E., Hadidi, A., 1998. Economic losses due to plant viruses. In: Hadidi, A., Khaterpal, R.K., Koganezawa, H. (Eds.), Plant Virus Disease Control. APS Press, St. Paul, MN, USA, pp. 1–13.
Wetzel, T., Candresse, T., Ravelonandro, M., Dunez, J., 1991. A polymerase chain reaction assay adapted to plum pox virus detection. J. Virol. Methods 33, 355–365.
Wetzel, T., Candresse, T., Macquaire, G., Ravelonandro, M., Dunez, J., 1992. A highly sensitive immunocapture polymerase chain reaction method for plum pox virus detection. J. Virol. Methods 39, 27–37.
Williams, B.A., Gwirtz, R.M., Wold, B.J., 2004. Genomic DNA as a cohybridization standard for mammalian microarray measurements. Nucl. Acids Res. 32, e81.
Williams, B.A., Gwirtz, R.M., Wold, B.J., 2006. Genomic DNA as a general cohybridization standard for ratiometric microarrays. Methods Enzymol. 410, 237–279.
Yang, Y.H., Speed, T., 2002. Design issues for cDNA microarray experiments. Nat. Rev. Genet. 3, 579–588.
Yang, D.-H., Barari, M., Arif, B.M., Krell, P.J., 2007. Development of an oligonucleotide-based DNA Microarray for transcriptional analysis of *Christone fumiferana* nucleopolyhedrovirus (CfMNPV) genes. J. Virol. Methods 143, 175–185.